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ASSESSMENT OF APOPTOSIS IN RATS CHRONICALLY EXPOSED TO ORGANIC SOLVENTS

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Abstract. Measuring the effect of various chemical substances on murine spleen and thymus is an usual method used in immunotoxicology. Apoptosis is a form of cellular death which can be triggered by toxic insults. The characteristic morphological alterations of apoptotic cells were assessed using acridine orange, a DNA-intercalating dye. We compared thymus and spleen apoptotic index (percentage of apoptotic cells) in rats chronically exposed to various organic solvents: toluene, xylene, acetone, a mixture containing 30% toluene, 30% xylene and 40% acetone and a mixture containing 15% benzene and 85% toluene. All the solvents used in the experiment induced apoptosis in splenic and thymic cellular suspensions. Acetone, either alone or in mixture, was the most powerful apoptosis inducer.
Key words: apoptosis, acridine orange, organic solvents

Rezumat. Determinarea efectului pe care îl au diverse substanţe chimice asupra splinei şi timusului murin reprezintă o metodă uzuală în imunotoxicologie. Apoptoza este o formă de moarte celulară care poate fi declanşată de substanţele toxice. Modificările caracteristice ale morfologiei celulelor apoptotice au fost evidenţiate folosind acridin orange, un colorant care se intercalează în ADN. Am comparat indicele apoptotic (procentul de celule apoptotice) din timusul şi splina şobolanilor expuşi cronic la diverşi solvenţi organici: toluen, xilen, acetonă, un amestec conţinând 30% toluen, 30% xilen şi 40% acetonă şi un alt amestec conţinând 15% benzen şi 85% toluen. Toţi solvenţii folosiţi în experiment au indus apoptoza în suspensiile celulare splenice şi timice. Acetona, fie singură fie în amestec, a fost cel mai puternic inductor de apoptoză.
Cuvinte-cheie: apoptoza, acridin orange, solvenţi organici

INTRODUCTION

It is considered that there are two phenomena which lead to cellular death: necrosis and apoptosis. Necrosis represents a passive process that occurs in response to strong injury (1-4) and generally is characterized by swelling of the cell and its nucleus, loss of electrolyte balance and, eventually, rupture of the plasma membrane (5). Apoptosis (programmed cell death) is by far a more intricate process since it represents an active way by which the cell itself commands and executes the program leading to its own destruction.

The programmed cell death is a paramount mechanism contributing to the maintenance of immunological homeostasis. It occurs whenever the body is exposed to non-lethal doses of xenobiotic chemicals (6-8) and it

succeeds to eliminate the damaged cells without inducing an inflammatory reaction.

Due to the development of immuno-toxicology, we know today that the environmental chemicals can affect the immunological defense system (9). Many reviews have highlighted the xenobiotic-induced apoptosis on immune-competent cells.

Preservatives added to detergents, cosmetics or vaccines were shown to modulate the apoptotic machinery at low concentrations (1). Ethanol exposure of mice led to an increased percentage of apoptotic splenocytes (10) and detectable thymocyte apoptosis was observed in C57BL/6 mice after injection of a single dose of 2,3,7,8-Tetrachlorodibenzo-para-dioxin (TCDD) (11). Also, it is now recognized that tobacco smoke (12) and Diesel exhaust particles (13) induce apoptosis in human monocytes.

Histologically, apoptotic cells have some characteristic features such as nucleus condensation and fragmentation, membrane blebbing and segmentation of the cell into apoptotic bodies (14). Acridine orange, a commonly used DNA-intercalating fluorescent dye, allows us to distinguish apoptotic cells by means of fluorescence microscopy.

We choose this method in order to detect apoptosis in thymocyte and splenocyte suspensions of rats chronically exposed to organic solvents thus aiming to assess the immuno-toxicity of these solvents.

MATERIALS AND METHODS

Chemicals

All the organic solvents utilized in this experiment were of chromatographic grade and were obtained from Sigma (benzene), UCP (xylene) and Chimopar (acetone and toluene).

Animals

42 white male Wistar rats, weighing 250±30 g, were purchased from Victor Babes Institute in Bucharest and were housed under constant, identical conditions of temperature (18^0-19^0C) and humidity (40-60%), receiving a natural diet and water *ad libitum*.

Experiment

The animals were distributed in 7 groups (6 rats/group). Each group was intraperitoneally injected with one of the following substances or mixtures: toluene (T), xylene (X), acetone (A), mixture 1 (30% T, 30% X, 40% A) and mixture 2 (15% benzene, 85% T). Each solvent or mixture was administered in a dose equal to the current occupational exposure limit, as follows: 850 mg T/kg, 1500 mg X/kg, 1500 mg A/kg, 1200 mg mixture 1/kg, 500 mg mixture 2/kg.

The injected dose was 0.5 ml (in sun-flower oil)/100 g body weight and was administered weekly during a 3 months period. Only oil (0.5 ml/100 g body weight) was administered to one control group and another control group included untreated rats.

At the end of the 3 months of treatment the rats were sacrificed; then

the thymus and the spleen were aseptically removed.

Apoptosis assay

The thymocyte and splenocyte apoptosis index was determined using the staining with acridine orange (Sigma) as described by Coligan (15). In order to obtain cell suspensions, the lymphoid organs were teased through a stainless steel mesh in RPMI1640 culture medium using a plastic syringe plunger.

The monocellular suspensions were washed three times at 1400 rpm, 10 min. at room temperature; resuspended in culture medium and counted in a Burker chamber.

2 x 10^4 cells/100 µl phosphate buffered saline were stained with acridine orange (final concentration of 3 µg/ml) in dark for 10 min. at room temperature; then were smeared on slides (DAKO ChemMate, BioTek Solutions) by centrifugation at 1000 rpm for 5 min. in a cytocentrifuge (Hettich, Universal 32R).

Five hundred cells from 10 microscope fields were counted using a fluorescence microscope (Nikon) equipped with a filter combination similar to that used for fluorescein (excitation = 455-496 nm, dichromatic light = 505 nm, barrier filter = 510 nm). The apoptosis index was expressed as the percentage of apoptotic cells identified among the counted cells.

Statistical analysis

The data are presented as mean percentage of the 6 rats from each group and the statistical significance was established by Student's *t* test.

Percentages of apoptosis index obtained in the oil-treated control group and those determined in the untreated-control group were similar, so they were considered as a single group.

RESULTS AND DISCUSSION

A major focus of immunotoxicology is the detection and evaluation of undesired effects of substances by means of tests on rodents (16).

Among various components of the immune system, the thymus and spleen are very sensitive to toxicity, a condition particularly illustrated by the rapidly dividing thymocytes (17).

The programmed-cell-death (apoptosis) is a process involved in the removal of potentially harmful cells which are phagocytized prior to the release of pro-inflammatory intracellular contents.

In this study we investigated the immunotoxic effect of three organic solvents: toluene, xylene, acetone – either used independently or in a blended solution – as well as a mixture of benzene and toluene (table 1).

Thus, we evaluated the apoptosis in thymocyte and splenocyte suspensions from rats chronically exposed to the above-mentioned solvents by staining the cells with acridine orange. The fluorescence microscopy analysis allowed us to distinguish between the live, non-apoptotic cells and the apoptotic ones.

Table 1. Thymocytes and splenocytes apoptosis in rats after 3 months of exposure to solvents

Solvents	Apoptosis index (%)[a]	
	Thymus	Spleen
Toluene	5.4	5.1*
Xylene	21.5*	9.4*
Acetone	34.0*	17.0*
Mixture 1	28.3*	16.5*
Mixture 2	9.1*	3.1*
Control	4.3	0.8

[a]Mean percentage of 6 animals/group; * $p < 0.05$

Because of the chromatin condensation which occurs in the early stage of apoptotic process, the apoptotic cells stained with acridine orange have increased green fluorescence of nuclei (fig. 1) as compared with those of live, non-apoptotic cells (fig. 2).

Our results presented in table 1 showed that all the solvents investigated led to an increase of apoptosis indexes in both lymphoid organs when compared with controls. When there were taken into consideration the differences between percentages of the solvents we noticed that acetone was the most powerful inducer of apoptosis.

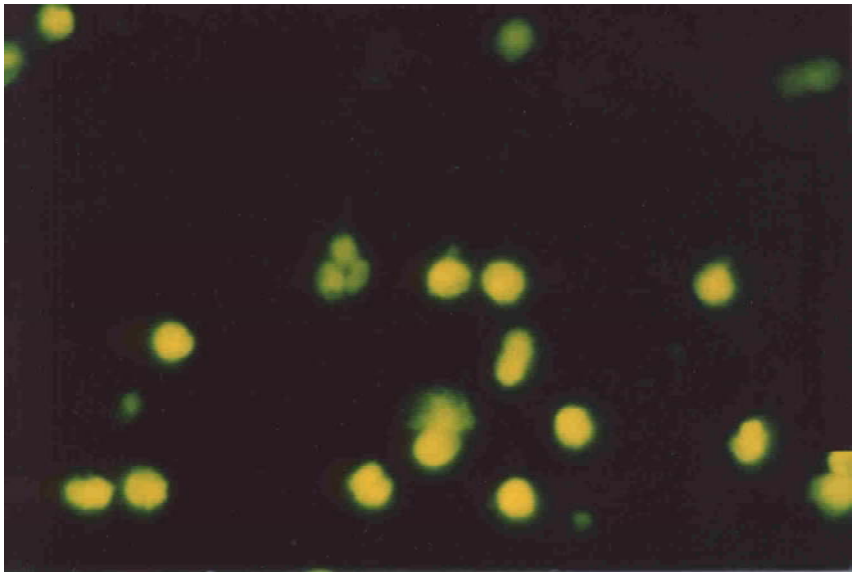

Fig. 1 Apoptotic thymocytes (bright green nuclei) in a rat chronically exposed to acetone

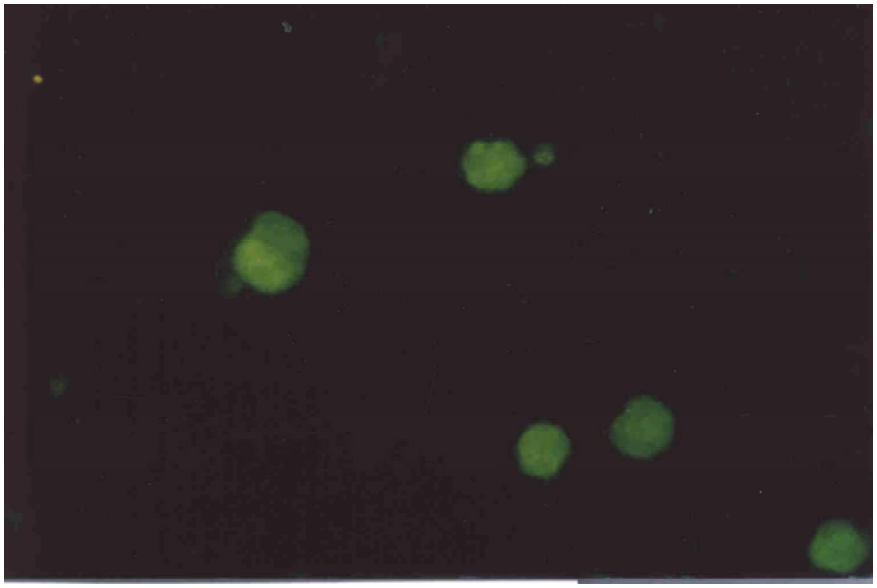

Fig. 2 Live, non-apoptotic thymocytes in a control rat

CONCLUSION

Our results represent an important contribution to the immunological studies concerning the organic solvents. We demonstrated by means of fluorescence microscopy that some organic solvents could induce apoptosis of the immune-competent cells.

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